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Formation of Quinonoid Structures in Laccase-Mediator Reactions

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This paper is dedicated to
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A softwood kraft pulp (kappa # 71.4) was subjected to a series of laccase-mediator treatments using 1-hydroxybenzotriazole (HBT), *N*-acetyl-*N*-phenylhydroxylamine (NHA), and violuric acid (VA). Based on the experimental conditions used in this study, the highest delignification response was observed with VA. Losses in brightness were observed after all three LMS systems, and were attributed to the formation of quinonoid structures. The residual lignins were isolated and derivatized with trimethylphosphite. The ^{31}P NMR spectral analyses confirmed the formation of quinones from $LMS_{VA,NHA,HBT}$. The decrease in quinones after an LMS(E) could be attributed, in part, to a Michael addition of OH^- to quinones.

Introduction

Over the last two decades, research efforts in pulping and bleaching have largely focused on environmental issues. As these concerns continue to be addressed, new research opportunities are developing. One area of active research focuses on improving pulp yields from pulping and bleaching operations [1-7]. The benefits in enhancing pulp yields are fourfold, including improved wood utilization practices, reduced pulp manufacturing capital costs, reduced operating costs, and improved profitability. An attractive approach for improving pulp yields consists of halting the kraft cook prior to reaching the terminal phase. In the terminal phase of pulping, the selectivity between lignin removal and carbohydrate degradation is significantly reduced resulting in loss of pulp carbohydrates. Stopping a kraft cook prior to the terminal phase reduces carbohydrate losses but yields a pulp with high lignin content (pulp kappa number of 40-50). A promising strategy for removing the lignin from such pulps prior to bleaching consists of employing a single- or a double-oxygen stage. Several research groups have reported that pulp yields can be increased in the range of 2-6% by employing this approach [2,5,6].

Recently, we have begun investigating the potential of employing lignin degrading enzymes, more specifically, laccase-mediator systems (LMS), to delignify high kappa kraft pulps [8,9]. Laccase has been shown to effectively oxidize phenolic compounds and phenolic lignin model compounds [10-15]. However, laccase alone is ineffective in delignifying pulp fibers [16]. This inefficacy is attributed to the size of the enzyme and, hence, to its inability to diffuse in a pulp fiber and oxidize the lignin [17]. The limitations of diffusion were shown to be circumvented by the addition of low molecular weight compounds, also known as mediators. Bourbonnais et al. first demonstrated this approach when they reported that laccase in the presence of 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonate) (ABTS) could delignify kraft pulps [18]. In addition, based on model compound studies, it was shown that the specificity of the laccase-mediator system could be expanded to non-phenolic substrates [19-21]. The proposed mechanism for this mediator assisted biodelignification process involves laccase oxidizing ABTS and, in turn, the oxidized ABTS diffuses into the pulp fiber and reacts with the lignin. The reduced ABTS is then reoxidized by laccase. Since then, a host of alternative mediators have been reported in the literature [22-24]. Some of the more effective mediators are N-hydroxybenzotriazole (HBT), violuric acid (VA), and *N*-acetyl-*N*-phenylhydroxylamine (NHA). Typically, these mediators have been employed with laccase on low-lignin content kraft and sulfite pulps, as well as on lignin model compounds [24-36].

The ability of these LMS treatments to remove lignin from high-lignin content pulps has remained largely unexplored. We have recently demonstrated that an LMS treatment, using HBT or NHA as mediators, can remove significant amounts of lignin from high-kappa kraft pulps [8,9]. NMR analysis of the residual lignin in the pulp after an LMS treatment indicated that the bio-delignification treatment extensively oxidizes C-5 noncondensed phenolic lignin structures, whereas C-5 condensed phenolic lignin structures were overall resistant to oxidation. In addition, side chain oxidation did occur on the propane-linking unit of lignin. The primary product detected from these oxidative treatments has been the formation of carboxylic acid groups [8,9].

The presence of quinone groups in an LMS treated pulp has been frequently proposed [8-9,28,30,36-37]. Lignin model compounds studies with laccase indicate that this can occur [38]. Recently, Poppius-Levlin et al. [28,39] presented FT-IR data suggesting that the residual lignin from LMS treated pulp has an enriched level of quinonoid structures.

The formation of quinones in LMS treated pulps could readily explain the substantial increases in brightness when a kraft pulp is first subjected to LMS and then treated with alkaline hydrogen peroxide [8-9,36-37]. It is well established that alkaline hydrogen peroxide readily reacts with para and ortho-quinones [40,41]. The removal of these intensively colored bodies from kraft pulp with alkaline peroxide would significantly improve the brightness of the pulp. The purpose of this study was to determine the relative amounts of quinones in residual lignins isolated from a softwood high-kappa kraft pulp before and after LMS treatments, using HBT, NHA, and VA as mediators.

Experimental

Methods and Materials

Chemicals. All chemicals were purchased from Aldrich Co., Milwaukee, WI, and used as received, except for *p*-dioxane, NHA, and laccase. *p*-Dioxane was freshly distilled over $NaBH_4$ prior to using it for lignin isolation experiments. NHA was synthesized in accordance with Oxley's method [42]. Laccase from *Trametes villosa* was donated by Novo Nordisk Biochem, Franklinton, NC.

Furnish. The softwood kraft pulp employed in this study originated from a 25-year-old *Pinus taeda* tree that was donated by Union Camp (now International Paper), Savannah, GA. The chips were cooked at Potlatch Corp. facilities in Cloquet, MN, to an H-factor of 573 using 18.5% active alkali. The

pulp was thoroughly washed, screened, centrifuged, fluffed, and stored at 4°C. Prior to executing the experimental design called for in this study, the pulp was Soxhlet extracted with acetone for 24 hours and then thoroughly washed with distilled water to remove the residual acetone.

Hexenuronic acid in pre-acetone extracted brownstock. The content of hexenuronic acids in the brownstock was indirectly measured in accordance with a modified procedure reported by Vuorinen et al. [43]. In brief, a 1000-mL round bottom flask was charged with 25 g of pulp (o.d. basis). The pulp consistency was adjusted to 3% by adding distilled water. The pH was then lowered to 3 using 4.0 N sulfuric acid. The slurry was refluxed for three hours at 100°C. The change in kappa number before and after the treatment was then determined and served as an indirect measurement of hexenuronic acids (see **Table I**).

Table I. Changes in Kappa # After Acid Treatment of Brownstock

Replicate #	Initial Kappa	Final Kappa	% Change
1	73.4	71.5	2.6
2	73.4	71.9	2.0

Laccase assay. Laccase activity was measured by monitoring the rate of oxidation of syringaldazine. One unit of activity (U) was defined as the change in absorbance at 530 nm of 0.001 per minute per milliliter of enzyme solution, in a 100 mM potassium phosphate buffer (2.2 mL) and 0.216 mM syringaldazine in methanol (0.3 mL, pH 6.7). The procedure was carried out at 23°C. The activity of the laccase was 1.87E + 06 U/mL of enzyme solution.

Laccase-mediator delignification procedure (LMS). A 2000-mL capacity Parr reactor equipped with a stirrer, a pressure gauge, a heating mantle, and connected to a Parr 4842 temperature controller was charged with 60 g of o.d. fibers. The pulp consistency was adjusted to 5% with distilled water. The slurry was then heated to 45°C and was maintained at this temperature throughout the incubation period. VA (4.4 mmol/10g of o.d. pulp, or NHA, or HBT) was then added to the heated slurry. Subsequent to mixing the slurry (approx. 5 minutes), the pH was adjusted to 4.5 with glacial acetic acid or saturated sodium bicarbonate solution. Laccase (93,500 U, or 0.05 mL of enzyme solution/g of o.d. fiber) was added, and the reactor was sealed and pressurized with oxygen to 145 psig. After a mixing period of 1 hour, the pulp was removed from the reactor and thoroughly washed with distilled water (12 L

per 10 g of o.d. pulp). The treated and washed pulp was either followed by a subsequent alkaline extraction stage or simply used as is.

Alkaline extraction stage (E). Alkaline extractions were performed in 4 mm-thick Kapak heat sealable pouches for 1 hour, at 80°C, and 10% consistency. All E treatments employed 2.5% charge of NaOH.

Control experiments in the absence of laccase (MS). Control experiments were performed in the absence of laccase and in the presence of VA, HBT, and NHA. These treatments were carried out in accordance with the laccase-mediator delignification procedure described above, except no laccase was employed.

Pulp characterization. The brownstock, MS, LMS, and LMS(E) pulps were characterized for kappa number and brightness in accordance with standard TAPPI Standard Methods T236-cm85 and T452-om98, respectively.

Isolation of residual lignins. The isolation of residual lignins was carried out following standard literature methods [44-46]. In summary, a 5000-mL three-necked round bottom flask was charged with 50 g of o.d. pulp and the consistency was adjusted to 4% by adding a 0.10 N HCl 9:1 *p*-dioxane:water solution. The slurry was then refluxed for two hours under an argon atmosphere. The pulp was filtered and the filtrate was filtered through celite, neutralized, and concentrated under reduced pressure to approximately 10% of the original volume. Water (approx. 400 mL) was added and the mixture was concentrated again under reduced pressure to remove the last traces of *p*-dioxane. The solution's pH was then adjusted to 2.5 with 1.00 N HCl. The precipitated lignin was collected, washed several times, and freeze-dried. Lignin yields ranged from 46.3 to 49.0%. Lignin yields were calculated as follows: % lignin yield = {mass of lignin isolated/ (initial kappa of brownstock x 0.15)}x100. The calculated lignin yields were corrected for initial hexenuronic acid groups content.

Derivatization of residual lignins with trimethylphosphite (TMP). Derivatization of residual lignins with trimethylphosphite was performed in accordance with Zawadzki's method [47,48]. In brief, a 30 mg sample of lignin previously dried at 40°C under vacuum for 24 hours was treated with 500 µL of 50% TMP/DMF (v/v) under an argon atmosphere for 7 days. Subsequent to the incubation period, excess trimethylphosphite was removed by first adding 250 µL of DMSO and then placing the lignin solution under vacuum at 45°C until the sample was nearly dry (approx. 6 hours). The treated

lignin samples were then dissolved in 500 μL 60% of DMSO-d_6/pyridine (v/v) containing tri-meta-tolylphosphate (0.7 mg/mL) and chromium–acetylacetonate (0.9 mg/mL). Deionized water (5 μL) was then added and the lignin solution was allowed to mix for 12 hours prior to acquiring the ^{31}P NMR spectrum.

^{31}P NMR of derivatized residual lignins. ^{31}P NMR spectra of derivatized lignins were acquired using a 90° pulse, a 5-second pulse delay, inverse-gated broad-band proton decoupling and 1000 scans per spectrum (approx. 1 hr 36 min total acquisition time) [47,48]. All ^{31}P NMR spectra were recorded on a DMX 400 MHz Bruker spectrometer.

Results and Discussion

Extent of Delignification and Brightness

The delignification and brightness responses of laccase-mediator systems employing HBT, NHA, and VA on a softwood kraft pulp (kappa # 71.4) were

Table II. Kappa and TAPPI Brightness for a softwood kraft pulp treated with MS[a], LMS[b] and LMS(E)[c] using HBT, NHA, and VA as mediators[d].

Pulp/Treatment	Kappa #	St.dev	TAPPI Brightness	St.dev
Brownstock	71.4	0.19	18.4	0.11
MS_{NHA}	71.3	0.11	18.5	0.20
MS_{HBT}	71.0	0.18	18.7	0.33
MS_{VA}	71.2	0.08	18.5	0.15
LMS_{NHA}	67.0	0.29	7.8	0.43
LMS_{HBT}	65.3	0.11	11.6	0.45
LMS_{VA}	53.6	0.09	9.8	0.39
$LMS_{NHA}(E)$	58.4	0.37	10.7	0.21
$LMS_{HBT}(E)$	57.4	0.01	15.5	0.35
$LMS_{VA}(E)$	45.1	-	13.7	0.37

[a]MS treatment in the absence of laccase but in the presence of mediator.
[b]LMS treatment in the presence of both laccase and mediator.
[c]LMS(E) treatment in the presence of both laccase and mediator and followed by an alkaline extraction stage (E).
[d]see experimental section for details.

evaluated before and after an alkaline extraction stage (E). In addition, a series of control experiments in the absence of the laccase were carried out. The kappa and brightness measurements relative to the initial brownstock are summarized in **Table II**.

The results clearly indicate that in the absence of laccase and in the presence of the mediator only, delignification did not occur. In addition, previous LMS studies have demonstrated that the delignification response of a laccase treatment in the absence of a mediator is insignificant [16,26]. Hence, both the mediator and the laccase must be present in order to achieve delignification. Based on the experimental conditions employed in this study, VA was a superior mediator with respect to HBT and NHA on this high-kappa kraft pulp. The extent of delignification of both NHA and HBT was comparable.

It is well known that a high content of hexenuronic acids (HexA) has an adverse impact on the kappa number since HexA consume potassium permanganate [43]. As summarized in **Table I**, the change in kappa number after the acid stage was approx. 2%, implying that the HexA content is insignificant and that the kappa numbers in this study were a good reflection of the lignin content.

Accompanying the LMS delignification, the treated pulps suffered a loss in brightness. The brightness data shown in **Table II** indicate that the LMS treatment always darkens the pulp with respect to the brownstock. This effect was most significant with NHA and VA. The extraction stage with sodium hydroxide improved the final brightness of the LMS treated pulps relative to the brownstock; however, it never exceeded the initial brightness. Based on our previous studies [9,36], we have shown that this darkening effect can be further alleviated with the reinforcement of the extraction stage with peroxide, and with peroxide and oxygen. This effect is consistent with the proposed quinone chemistry of an LMS stage.

Quinone Content. The role of quinones in the observed LMS delignification chemistry was explored by isolating the residual lignin from the SW kraft brownstock, and after the MS, LMS, and LMS(E) treatments, as described in the experimental section. The combined content of ortho- and para-quinones was examined using a trimethylphosphite derivatization procedure and ^{31}P NMR. Studies by Zawadzki and Ragauskas [48-51], Argyropoulos and Zhang [52], and Zhang and Gellerstdedt [53] have shown that trimethylphosphite can readily be used to tag ortho- and para-quinones and after hydrolysis yield a stable phosphate ester adduct. This adduct is detected via ^{31}P NMR experiments and is a means to establish a semi-quantitative relationship of the quinone content. The combined ortho- and para-quinone data are presented in **Figure 1**.

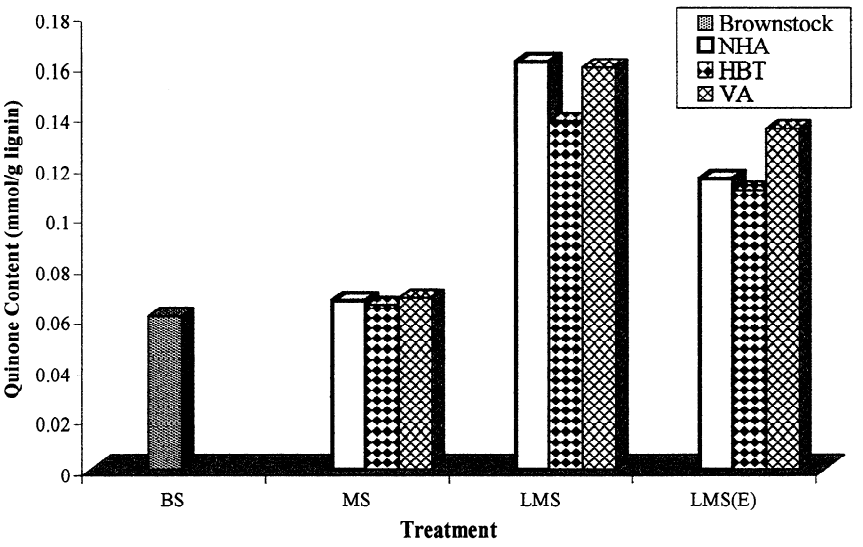

Figure 1. Semiquantitative quinone content of residual lignins isolated from the brownstock (BS) and after MS, LMS, and LMS(E) treatments using NHA, HBT, and VA as mediators.

The experimental results indicate that the content of quinone structures in the brownstock is minute. This value is comparable to that reported by Zawadzki [50]. Treatment of the pulps in the presence of mediators and oxygen failed to introduce any further quinones into the residual lignins.

Repeating these experiments in the presence of both laccase and mediator led to an approximate 2.7-fold increase in detectable quinone structures when NHA and VA were used. The relative trend also suggests that the content of quinones was lower when HBT was employed.

The subsequent alkaline extraction stage reduced the quinone content, on average, by approximately 21%. The loss of quinones during the alkaline extraction stage can be attributed to the reactivity of NaOH with such structures. The nucleophilic addition of OH^- to quinonoid structures can result in increased solubility. This type of chemistry can lead to the formation of hydroxy substituted catechols *via* a Michael addition of hydroxide anions and also to alpha-hydroxy-carboxylic acid cyclopentadiene structures (see Figure 2). The latter structures are postulated to stem as a result of a nucleophilic addition of OH^- followed by a benzylic acid rearrangement [54].

Despite the loss in quinone structures subsequent to the extraction stage, the data suggest that the residual lignin still contained approximately 50% more quinone structures than the brownstock. One possible explanation for

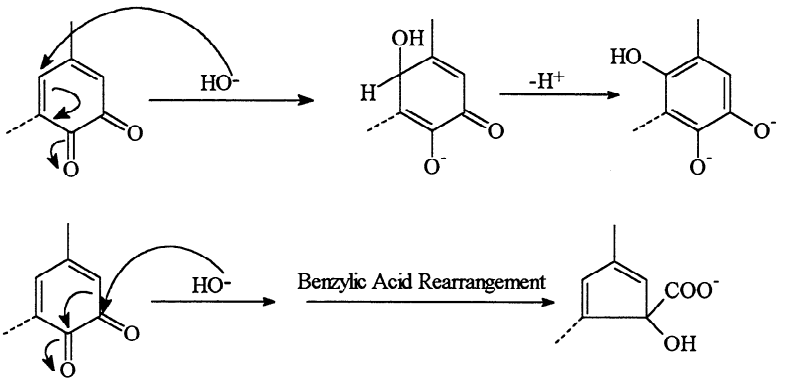

Figure 2. Proposed sites of addition of hydroxide anions to quinone structures [54,55].

this observation could be attributed to the proposed propensity of *o*- and *p*-benzoquinones to undergo condensation reactions leading to the formation of bi-phenyl linkages [55]. As a result, the solubility of such structures may be adversely affected. Another possible explanation may be linked to the ability of catechols to readily be oxidized back to quinone structures.

Conclusions

In summary, this study provides some of the first spectroscopic data that establishes conclusively the formation of quinones in LMS and LMS(E) treated softwood kraft pulps. The data provide an explanation, in part, for the darkening of kraft pulps after an LMS stage and its subsequent partial brightening after an LMS(E) stage. The observed formation of quinones after an LMS stage is also consistent with the reported brightness benefits of alkaline peroxide bleaching of LMS treated pulps. The formation of quinones and the darkening effect of pulps are important aspects of the chemistry of LMS delignification. This issue will need to be addressed and further understood if LMS technology is to be implemented commercially.

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